Remarks

After amendment, claims 1-2, 5-16 and 46-47 remain pending in the present application, after canceling *without prejudice* claims 17-45 pursuant to the Examiner's restriction requirement and Applicant's decision to prosecute the invention of group I, namely claims 1-16. Claims 3 and 4 are cancelled. It is anticipated that Applicants will file a divisional application which is directed to subject matter which has been cancelled from this application. The amendment to the claims has been made to clarify the originally filed claims and to expedite allowance of the instant application. An active step (e) was previously added to claim 1. Claims 1 and 12 have been amended to reflect the fact that the patient being diagnosed is at risk for the disease for which the diagnosis is being made and the biological sample is a urine, serum or plasma sample. No new matter has been added by way of this amendment.

The Examiner has objected to or rejected claims 1-16 variously under 35 U.S.C. §112, first and second paragraphs and §102(b). For the reasons which are set forth in detail herein, including a further explanation of the previously enclosed paper from Valmu, et al., *Glycobiology* 2006 Dec. 16(12):1207-18 (Valmu, et al.), which shows that the glycosylated hCG variant measured in the instant invention, ITA, which contains a substantial number of O-linked glycosyl linkages is *clearly* distinguishable from the N-linked glycosylated hCG variant measured by Kobota. This evidence clearly obviates the Examiner's rejection of the instant claims as being anticipated by Kobota. It is respectfully submitted that the instant claims are patentable over the disclosed prior art. Applicants shall address each of the objections/rejections to the claimed invention in the sections which appear hereinbelow.

The Rejection of Claims 1, 4-8, 10 and 11 under 35 U.S.C.§102(b)

The Examiner has maintained his rejection of claims 1-8 and 10-15 under 35 U.S.C. §102(b) as being anticipated by Kobata, *Biochemie,* 1988, 70: 1575-1585 ("Kobata"). It is the Examiner's continued contention that Kobata teaches a method of

measuring ITA in a urine sample and on the basis of the percentage of ITA compared to the total amount of hCG in the sample, detecting invasive trophoblast cells if the percentage of ITA in the sample is greater than 30% of the total amount hCG. Applicant respectfully traverses the Examiner's rejection. It is noted that the variant of hCG which is measured by Kobata is *not* the same as the variant measured by present invention.

Essentially Applicant's method is clearly patentable and *not* anticipated by the method of Kobata simply because Kobata teaches measuring a variant of hCG which is not ITA, as that term has been defined in the specification. In particular, Kobata is directed to measuring an N-linked glycosylated variant of hCG, not a glycosylated version of hCG containing O-linked glycosyl as in the present invention (note that ITA, unlike the Kobata glycosylated hCG, contains both N-linked and O-linked glycosyl groups, whereas Kobata contains exclusively N-linked groups). This is explained in great detail in the previously submitted Valmu, et al. article. Inasmuch as there are a number of glycosylated variants, it is the type of variant which will determine the accuracy of the assay and whether or not invasive trophoblast cells exist in a patient. In the case of Kobata, Kobata is measuring N-linked glycosylated versions of hCG, not ITA of the present invention, which is a hyperglycosylated variant of hCG which contains both N-linked and O-linked glycosyl groups.

Note that the definition of ITA, the O-linked glycosylated variant which is measured in the present invention, is set forth in the specification at page 5, in the second full paragraph. This is the variant which Applicant has focused on and to which the present invention is directed to measuring. This is ***not*** what Kobata is measuring. The two types of hyperglycosylated variants of hCG, i.e., those of Kobata and those of the present invention, are quite distinguishable. This is clearly presented in Valmu, et al. at page 1213 and in particular, in the first full paragraph bridging the first and second columns. That disclosure clearly evidences that Kobata is measuring an N-glycosylated variant of hCG, not the variant ITA of the present invention. Moreover, Valmu, et al. points out that the Kobata N-glycal hCG variant could not be detected by the mass-matching approach because the structures carry the same mass as ordinary biantennary N-

glycals. This structural feature of the hCG variant measured by Kobata makes its measurement difficult given its similarity in structure to related variants. This would also explain the relatively low accuracy of the Kobata method compared to the method of present application. It is clear from Valmu, et al. that the Kobata measured hCG variants are clearly distinguishable from ITA, the variant measured in the present invention. See Valmu, et al. also at page 1216, first full paragraph in the left column. Given the fact that the present invention and Kobata measure two distinguishable types of glycosylated hCG as evidenced by the teachings of Valmu, et al., the method of Kobata does not anticipate the present invention.

In contrast to the present method, Kobata *only* deals with and measures N-linked glycosylated hCG, not the O-linked glycosylated hCG which is measured in the present invention. Thus, the present invention clearly distinguishes over Kobata in measuring a different hCG variant (known as HhCG or ITA) than Kobata. Thus, because Kobata is not directed to the same or identical method as the present invention (because of the clearly distinguishable variants which are measured in the disclosure of Kobata vs. the present method), Kobata does not and *cannot* anticipate the present invention.

Valmu, et al. clearly shows that the O-linked glycosylated hCG variant ITA, which is measured in the present method, is distinguishable from the N-linked glycosylated hCG which is measured by Kobata, as discussed above. Not only does the enclosed paper show the distinction between the N-linked and O-linked glycosylated variants of hCG, but also points to the superiority of measuring ITA- which is the only significant and consistent change in choriocarcinoma. Thus, Kobata, clearly is directed to measuring a different hCG variant and the disclosed method clearly does not anticipate the present invention.

The Examiner continues to argue that Applicant, in order to distinguish Kobata, recites limitations which are not in the claims. Applicants respectfully traverse the Examiner's arguments. The fact that Applicant recites ***ITA*** in the claims ***is*** *a distinguishable limitation in the claims* because ITA, which contains O-linked glycosyl

groups as defined in the specification and as described in Valmu, et al., is distinguishable from the hCG variant measured in Kobata, which is directed to a different form of glycosylated hCG containing N-linked glycosyl groups. These hCG variants are clearly not the same and are clearly distinguishable. Given the fact that ITA and the hCG variant of Kobata are distinguishable, as evidenced by the clear description in Valmu, et al., the present claims are not anticipated by the art of record and the distinguishable limitation *is found* in the presently pending claims.

The Objection to the Drawings

The Examiner has objected to the drawings as referencing figure 5 on page 29 at line 14. Applicant, in order to address this objection, has amended the specification to delete any reference to figure 5.

The Objection to the Claims

The Examiner has objected to claims 9 and 16 as having informalities related to the spelling of placenta. Applicants have corrected the claims, thus obviating the Examiner's objection. In addition, Applicants have cancelled claim 5 to obviate the objection to that claim.

The Rejection of Claims 1-16 under 35 U.S.C. §112, Second Paragraph

The Examiner rejected original claims 1-16 under 35 U.S.C. §112, second paragraph as being incomplete for missing the step of determining the **total** amount of ITA in step1c and 12c. In order to obviate the Examiner's rejection, claims 1 and 12 have been amended to reflect the fact that the total amount of ITA is measured in step c of claims 1 and 12. It is respectfully submitted that the claims are now in conformity with the requirements of 35 U.S.C. §112, second paragraph.

Regarding the Examiner's query that the term "amount" renders claims 1 and 12 indefinite, Applicant's amendment of the claims to reflect the measurement of hCG and ITA, both of which terms are clearly defined in the specification, satisfies the requirements of 35 U.S.C. §112, second paragraph.

The Rejection of Claims 1-16 under 35 U.S.C. §112, First Paragraph

The Examiner has rejected claims 1-16 under 35 U.S.C. §112, First Paragraph for the reasons which are set forth in the office action at paragraph 12, on pages 6-7 and on page 13, paragraph 15. The Examiner contends *inter alia* that the claims are non-enabled because of the use of "in-house" assays to measure serum and urine samples and because the measurement of ITA as hyperglycosylated hCG is non-enabled and that the claimed amount of hCG cannot be measured pursuant to the present invention. Applicant respectfully traverses the Examiner's rejection.

It is respectfully submitted that the claimed invention is enabled. The invention as claimed relates to a method for measuring the total amount of intact hCG and ITA or the total amount of intact hCG and ITA plus the free beta form of hCG in a biological sample from a patient at risk for invasive trophoblastic disease or quiescent gestational trophoblastic disease as claimed and determining the amount of total intact hCG plus ITA or total hCG plus beta core plus ITA with the amount of ITA measured in the sample such that a diagnosis of invasive trophoblastic disease or quiescent gestational trophoblastic disease may be made. It is respectfully submitted that the present invention is enabled.

The measurement of the amount of intact hCG,, ITA and beta core hCG as defined in the specification in a urine, serum or plasma sample is well known in the art. The approach to measuring this amount may be through an immunoassay, or other commercially available hCG tests. Indeed many commercial assays measure same. This is discussed in significant detail in the specification *inter alia,* at page 7, fourth full paragraph of the specification, as well as on pages 8-10. Approaches for measuring hCG

in biological samples have been known for years and are well documented and routine in the art. The measurement of ITA as defined in the specification (containing both N-glycosyl linkages and O-glycosyl linkages as indicated in the specification) is also well known in the art. Analysis can be performed by any number of techniques as described in the present application at pages 8-10 and in particular, in an immunoassay using the B152 antibody which is specific for ITA (and the O-glycosyl linkages of ITA as explained in the Valmu, et al. paper). Other methods are readily adapted from prior art teachings. Thus the present invention relates to the measure of ITA as opposed to the N-glycosylated variant measured by Kobata and provides a well-known method available in the art including a specific monoclonal antibody B152 which is specific for ITA.

Thus, the present invention provides all of the well-known methods for measuring amounts of intact hCG, beta hCG and ITA in urine, serum and plasma samples from a patient. The remaining steps are also well-known and are enabled. It is respectfully submitted that the instant claims are clearly enabled.

Turning to the Examiner's observation that the originally filed claims were directed to a biological sample which could include cells and that measurements of the relevant polypeptides in those cells was non-enabled page 8, paragraph 13 of the office action), Applicant has amended the claims to reflect the fact that the biological sample is limited to urine, plasma or serum. It is respectfully submitted that the measurement of hCG, beta hCG and ITA as claimed in the biological sample is enabled, inasmuch as measurement of hCG, beta hCG and ITA in urine, plasma or serum is relatively facile and diagnosing the conditions as claimed flows directly and readily from those measurements. Inasmuch as the measurement of each of hCG, beta hCG and ITA as claimed is enabled, practicing the remaining steps of the claimed invention to determine the existence of the condition is also enabled and facile. The method now clearly reflects the fact that the condition is detecting the presence or absence of invasive trophoblast cells in a patient, not the sample.

Regarding the argument that the claimed method is not enabled, Applicant notes that the amount of intact hCG, ITA and optionally, beta hCG as claimed may be measured using any number of methods which are available in the art and are well described in the literature. In addition, as noted, commercial immunoassays may also be utilized to measure hCG and may be preferably used. These may be used directly or adapted with minor variation in order to obtain an amount of hCG in a sample. Antibodies are readily available commercially which may measure intact hCG and ITA, and optionally, beta hCG as defined in the specification. Regarding the measurement of ITA, the preferred method for measuring ITA in a sample is through the use of monoclonal B152, which is readily available. Thus, all of the components for practicing the invention are available and well known in the art, all of the steps are well known and practicing the method which simply relies on well known steps already known in the art using components which are readily available in the art evidences that the claimed method is clearly enabled.

Turning to the Examiner's rejection of claim 12 as failing to comply with the enablement requirement (page 10, paragraph 14), it is respectfully submitted that Applicant's have amended claim 12 to reflect an inventive method which is enabled. In particular, the method is directed to a method of diagnosing quiescent gestational trophoblastic disease in a patient at risk for that disease. Note that quiescent gestational trophoblastic disease is described in the specification on pages 6, second full paragraph, and is otherwise described on page 10 of the specification. It is respectfully submitted that the method of claim 12, which provides for steps related to determining levels of hCG and ITA and optionally beta hCG in a biological sample in a patient at risk for quiescent gestational trophoblastic disease is clearly enabled. Each of the delineated steps is well known, each of the components which may be used to measure individual values as described in the claim are readily available and performing the steps as set forth in amended claim 12 is routine. Claim 12 is enabled.

It is thus respectfully submitted that the presently pending claims are enabled.

Turning to the Examiner's rejection of the previously pending claims 1-16 that those claims fail to meet the requirements of 35 U.S.C. §112, first paragraph for failing to provide an adequate written description, Applicants respectfully traverse the Examiner's rejection. The present claims now adequately directed to measuring the amount hCG (intact hCG plus ITA alone or in combination with beta hCG) which is measured to provide the presently claimed method. That is now adequately described in the specification and set forth in the claims. A review of the claimed subject matter and the specification clearly evidences that the present invention is now in compliance with the requirements of 35 U.S.C. §112, first paragraph as related to the written description requirement. The present invention must be seen to be in compliance with the requirements of 35 U.S.C. §112, first paragraph.

For the above reasons, Applicant respectfully asserts that the claims set forth in the amendment to the application of the present invention are now in compliance with 35 U.S.C. Applicants respectfully submit that the present application is now in condition for allowance and such action is earnestly solicited.

Applicants have cancelled 2 claims, added two dependent claims and have previously cancelled 29 claims (5 independent). No fee is therefore due for the presentation of this amendment. A petition for a three month extension of time is enclosed as is the fee for the extension. If any fee is due or any overpayment has been made, please charge/credit Deposit Account No. 04-0838.

Should the Examiner wish to discuss the present application in an effort to advance its prosecution, the undersigned attorney may be reached at the telephone number set forth hereinbelow.

Respectfully submitted,

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Dated: July 10, 2007
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Site-specific glycan analysis of human chorionic gonadotropin β-subunit from malignancies and pregnancy by liquid chromatography—electrospray mass spectrometry

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Glycosylation is an important posttranslational modification in proteins, and aberrant glycosylation occurs in malignancies. Human chorionic gonadotropin (hCG) is a glycoprotein hormone produced in high concentrations during pregnancy. It is also expressed as particular glycoforms by certain malignancies. These glycoforms, which are called "hyperglycosylated" hCG (hCGh), have been reported to contain more complex glycan moieties. We have analyzed tryptic glycopeptides of the β-subunit of hCG of various origins by liquid chromatography (LC) connected to an electrospray mass spectrometer. Site-specific glycan structures were visualized by the use of differential expression analysis software. hCGβ was purified from urine of two patients with testicular cancer, one with choriocarcinoma, one with an invasive mole, two pregnant women at early and late gestation, from a pharmaceutical preparation and culture medium of a choriocarcinoma cell line. *N*-glycans at Asn-13 and Asn-30 as well as *O*-glycans at Ser-121, Ser-127, Ser-132, and Ser-138 were characterized. In all samples, the major type of *N*-glycan was a biantennary complex-type structure, but triantennary structures linked to Asn-30 as well as fucosylation of the Asn-13-bound glycan are increased in cancer-derived hCGβ. There were significant site-specific differences in the *O*-glycans, with constant core-2 glycans at Ser-121, core-1 glycans at Ser-138, and putative sites unoccupied by any glycan. Core-2 glycans at either Ser-127 or Ser-132 were enriched in cancer. The glycans of free hCGβ were larger and had a higher fucose content of Asn-13-linked oligosaccharides than intact hCG. This may facilitate the detection of this malignancy-associated variant by a lectin assay. Analysis of hCGh affinity purified with antibody B152 confirmed that this antibody recognizes a core-2 glycan on Ser-132.

Key words: antibody B152/cancer/hCG/hyperglycosylated hCG/mass spectrometry/N-glycosylation/O-glycosylation

Introduction

Glycosylation is one of the most important and common posttranslational protein modifications. It may affect both protein structure and function, but the impact of glycosylation is protein dependent. Because changes in protein glycosylation are associated with numerous diseases, including cancer (Hakomori, 2002), protein glycosylation is important in clinical research.

Recent advances in glycoproteomics, a field combining proteomics and glycomics, have been greatly influenced by methodological development. Especially mass spectrometry (MS) provides important structural information on glycoproteins (Harvey, 2001). MS analysis of glycoproteins based on the release of glycans either enzymatically or chemically provides structural glycan information, but the information on site occupancy is lost. Analysis of glycopeptides obtained by proteolytic digestion, typically with trypsin, provides site-specific glycan information, but this approach may be hampered by difficulties in the digestion of resistant glycoproteins. Furthermore, the analysis is often complicated by the heterogeneity of structurally related oligosaccharides occupying a single glycosylation site.

Human chorionic gonadotropin (hCG) is a glycoprotein hormone produced by placental trophoblasts and trophoblastic tumors. hCG is a heterodimer composed of α- and β-subunits (hCGβ). The α-subunit is shared with the other glycoprotein hormones, luteinizing hormone, follicle stimulating hormone, and thyroid stimulating hormone, whereas the β-subunit is specific for each hormone. hCG is expressed throughout gestation, and it occurs at high concentrations both in serum and in urine of pregnant women.

About one-third of the molecular weight of hCG consists of carbohydrates, and glycosylation is of structural and functional importance, affecting both the half-life in circulation and the signal transduction induced by this hormone (Lustbader *et al.*, 1998). The glycosylation pattern of hCG has been extensively studied. Two asparagine-linked carbohydrate units (*N*-glycans) on hCGβ at Asn-13 and Asn-30 (Carlsen *et al.*, 1973) have been shown to consist of biantennary, complex-type *N*-glycans with terminal sialic acids and a variable content of fucose (Kessler, Reddy, *et al.*, 1979; Weisshaar *et al.*, 1991). The site-specific *N*-glycan structures have been further characterized by MS using matrix-assisted laser desorption ionization (MALDI) technique (Laidler *et al.*, 1995; Jacoby *et al.*, 2000) and liquid chromatography (LC)–electrospray ionization (ESI) MS (Liu and Bowers, 1997). Four serine-linked oligosaccharides (*O*-glycans) attached to serine residues 121, 127, 132, and 138 have been shown to consist of monoantennary, so-called

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core-1 *O*-glycan structures with two sialic acids attached to each glycan (Kessler, Mise, *et al.*, 1979). However, core-2 type *O*-glycan at Ser-121 as well as putative glycosylation sites apparently not glycosylated has also been detected (Liu and Bowers, 1997). All MS studies on the glycan structure of hCG have been performed using pharmaceutical hCG preparations that have been partially purified from pooled urine from pregnant women.

Except in pregnancy, hCG is expressed by certain malignancies and especially by trophoblastic and testicular germ cell tumors, for which hCG is a sensitive marker (Stenman *et al.*, 2004). Furthermore, the concentration of free hCGβ in serum is often elevated in patients with nontrophoblastic cancers, and this is a sign of adverse prognosis (Alfthan, Haglund, Dabek, *et al.*, 1992; Alfthan, Haglund, Roberts, *et al.*, 1992; Stenman *et al.*, 2004). Various isoforms of hCG have been shown to be associated with malignancies. These include "nicked" hCG (hCGn), in which several peptide bonds in loop 2 of hCGβ are cleaved (Puisieux *et al.*, 1990; Birken *et al.*, 1999) as well as "hyperglycosylated" hCG (hCGh), which is the major form of hCG in trophoblastic cancer (Elliott *et al.*, 1997; Birken *et al.*, 1999) and early pregnancy (Kovalevskaya *et al.*, 2002). hCGh has also been associated with Down's syndrome (Cole *et al.*, 1999) and early pregnancy loss (Kovalevskaya *et al.*, 2002). Tumor-derived hCGh has been shown to contain increased amounts of triantennary *N*-glycans (Elliott *et al.*, 1997), abnormal biantennary *N*-glycans (Kobata and Takeuchi, 1999), and biantennary core-2 type *O*-glycans (Elliott *et al.*, 1997; Birken *et al.*, 2003).

Most studies on the carbohydrates of hCGh have been performed by chromatographic analysis of glycans released from the polypeptide, and this gives no site-specific structure information (Mizuochi *et al.*, 1983; Elliott *et al.*, 1997). An antibody to hCG, B152 (Birken *et al.*, 1999), recognizes a core-2 type *O*-glycan attached to Ser-132 and the surrounding peptide structures in hCGβ (Kovalevskaya *et al.*, 2002; Birken *et al.*, 2003). All clinical studies on hCGh have been based on the use of this antibody. No studies on site-specific oligosaccharide heterogeneity of cancer-derived forms of hCG, which are often lumped together under the term "hyperglycosylated" (Birken *et al.*, 2003), have been performed.

In this study, we have used novel differential expression analysis software to analyze site-specific glycan structures of hCGβ from various sources by LC–MS of tryptic protein fragments.

Results

Characterization of hCGβ purified from urine

hCG was purified from urine by serial immunoaffinity chromatography and gel filtration. Purified hCG was reduced and alkylated with 4-vinyl pyridine causing the dissociation of α- and β-subunits. hCGβ was isolated by reverse-phase (RP) chromatography, and its purity was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and further by LC–MS analysis of tryptic peptides. All the potential tryptic peptides (Table I) without attached glycans were visualized by LC–MS analysis with better than 20 ppm mass accuracy (Table II). The identity of the peptides was verified by LC–tandem mass spectrometry (MSMS) identification (data not shown). In addition to the predicted tryptic cleavage sites, additional cleavage sites were observed in peptides 5 and 9 (data not shown). The cleavage pattern of peptide 5 is in agreement with the proposed cleavage of loop 2 in the β-subunit in hCGn (Cole *et al.*, 1991), but in contrast to a previous report (Elliott *et al.*, 1997), the major sites cleaved were after amino acids 45 and 49, with some minor cleavage after amino acids 44, 46, and 48. In P9, the major cleavage occurred after Asn-77, but minor cleavage was also seen after amino acids 76, 78, 79, and 80.

Of the putative *N*-glycan-containing peptides P3 and P4 as well as the *O*-glycan-containing peptides P13, P14, and P15, some lacking the glycan were also observed (Table III), but with minor intensities.

When the *N*-glycans of hCGβ were enzymatically removed by peptide-*N*-glycosidase F (PNGase F), a mass shift from 37 to 29 kDa was observed by SDS–PAGE (data not shown). In LC–MS analyses of the tryptic digest, peptides P3 and P4 were detected at high intensity, with a mass adduction of 1 Da due to the conversion of asparagine to aspartic acids during PNGase F treatment (data not shown).

Site-specific N-glycan analysis of hCGβ

For glycan analysis, 3 pmol of hCGβ tryptic digest was subjected to capillary LC on an Atlantis dC18 column specially designed to retain hydrophilic peptides such as glycosylated ones. The LC–MS data generated were incorporated into DeCyder MS software, where two-dimensional (2D) intensity maps were visualized (Figure 1A), with each spot representing the intensity of a peptide in a certain charge state separated based on its *m/z* value and retention time, that is, hydrophilicity. The same peptide is represented by several charge states within the map. To identify tryptic peptides containing *N*-glycans, we compared the 2D LC–MS patterns using the DeCyder MS software PepMatch module to patterns of tryptic digests of hCGβ, from which the *N*-glycans had been enzymatically removed (Figure 1B). Two highly heterogeneous peptide patterns or "clouds" were observed for the N-glycosylated hCGβ peptides. In peptide-by-peptide analysis, peptides occurring at equal concentrations in the two digests (Figure 1C) were easily separated from peptides present only in the N-glycosylated molecule (Figure 1D). The identity of the differentially expressed peptides was studied by LC–MSMS fragmentation analysis (Figure 1E), which revealed sugar-specific oxonium ions (*m/z* 204 for *N*-acetylhexosamine (HexNAc), 274 for *N*-acetylneuraminic acid (NeuAc)-H_2O, 292 for NeuAc, 366 for Hex-HexNAc, and 657 for NeuAc-Hex-HexNAc) as a result of oligosaccharide dissociation. In addition, a clear fragmentation profile of the attached oligosaccharide portion of the glycopeptide was seen. Minor peptide-derived y-ions could also be detected in some peptide fragmentations facilitating differentiation between the P3 and P4 containing glycans (Figure 1A). The deglycosylation of the Asn-30-attached oligosaccharides from P4 was incomplete in part of the peptides.

Table I. Amino acid sequence of hCGβ and peptides derived by trypsin digestion

1	S	K	E	P	L	R	P	R	C	R	P	I	N	A	T	L	A	V	E	K	E	G	C	23
24	P	V	C	I	T	V	N	T	T	I	C	A	G	Y	C	P	T	M	T	R	V	L	Q	46
47	G	V	L	P	A	L	P	Q	V	V	C	N	Y	R	D	V	R	F	E	S	I	R	L	69
70	P	G	C	P	R	G	V	N	P	V	V	S	Y	A	V	A	L	S	C	Q	C	A	L	92
93	C	R	R	S	T	T	D	C	G	G	P	K	D	H	P	L	T	C	D	D	P	R	F	115
116	Q	D	S	S	S	S	K	A	P	P	P	S	L	P	S	P	S	R	L	P	G	P	S	138
139	D	T	P	I	L	P	Q																	145

The theoretical trypsin cleavage sites are indicated by vertical lines, the four glycosylated amino acids, Asn-13, Asn-30, Ser-121, Ser-127, Ser-132, Ser-138, are in boldface type. The most C-terminal GluC cleavage site is indicated by a dashed vertical line.

Table II. Observed *m/z* values, charge states, and deduced masses of tryptic peptides derived from 4-vinyl pyridine-alkylated hCGβ purified from the urine of a testicular cancer patient. Peptide sequences assigned for each observed mass are indicated by amino acid positions, and the theoretical mass of the assigned sequence is shown

Peptide	Position (amino acids)	Observed *m/z*[d]	Charge state	Deduced *m* (Da)	Theoretical *m* (Da)
P1 + P2	1–8	491.787	2	981.558	981.572
P3[a,c]	9–20				1418.770
P4[a,c]	21–43				2852.298
P5[c]	44–60	658.692	3	1973.052	1973.092
P5 + P6[c]	44–63	782.091	3	2343.249	2343.288
P7	64–68	651.342	1	650.334	650.339
P8[c]	69–74	747.392	1	746.384	746.390
P9[c]	75–94	592.796	4	2367.152	2367.169
P9 + P10[c]	75–95	631.817	4	2523.236	2523.270
P11 + P12[c]	96–114	742.321	3	2223.939	2223.968
P12[c]	105–114	637.274	2	1272.532	1272.556
P13[b]	115–122				884.388
P14[b]	123–133				1104.593
P15[b]	134–145				1233.660

[a]N-Glycosylation site.
[b]O-Glycosylation site(s)
[c]Ethylpyridyl-modified cysteines.
[d]At highest intensity.

In the LC–MS analysis, glycopeptides containing Asn-13-linked oligosaccharides were mainly preset as $[M + 3H]^{3+}$ and $[M + 4H]^{4+}$, with minor intensities of smaller peptides as $[M + 2H]^{2+}$. Ion counts of all different charge states of the same peptide were summarized to calculate the intensity of each glycopeptide. The masses of the oligosaccharides attached to P3 were calculated by subtracting the theoretical polypeptide portion mass of 1418.8 Da from the observed deconvulated masses of glycosylated peptides. The sugar structures were thus derived by the mass-matching approach (Figure 2). The same approach was used to calculate the oligosaccharides attached to Asn-30 in P4. Owing to their larger mass, P4 glycopeptides appeared in the LC–MS analysis mainly as $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$, with minor intensities as $[M + 3H]^{3+}$. Analysis of the Asn-30-attached oligosaccharides was complicated because Met-41 in P4 occurred both in native and in oxidized forms, and the intensities of these needed to be summed together (Figure 3). Interestingly, incomplete deglycosylation of P4 with PNGase F was observed in P4 with oxidized Met-41 (Figure 1B).

The N-linked oligosaccharides attached to Asn-13 and Asn-30 in hCGβ purified from the urine of a pregnant woman at 35 weeks of pregnancy, from the urine of a patient suffering from invasive mole, and from the urine of a patient with testicular cancer are shown in Figures 2 and 3. The relative intensities, which roughly represent the relative abundance of different glycan structures present, are shown with a value of 100 for the most intense component. Intensities of smaller glycan structures might be slightly increased relative to sialylated structures because of possible sialic acid release in mass spectrometric analysis. To simplify the picture of the enormous oligosaccharide heterogeneity, glycan structures with a relative intensity <15 in all three samples are omitted from the figures, but they were taken into account for the calculation of the proportion of all oligosaccharide structures shown in Tables III and IV. In addition to the structures shown, some

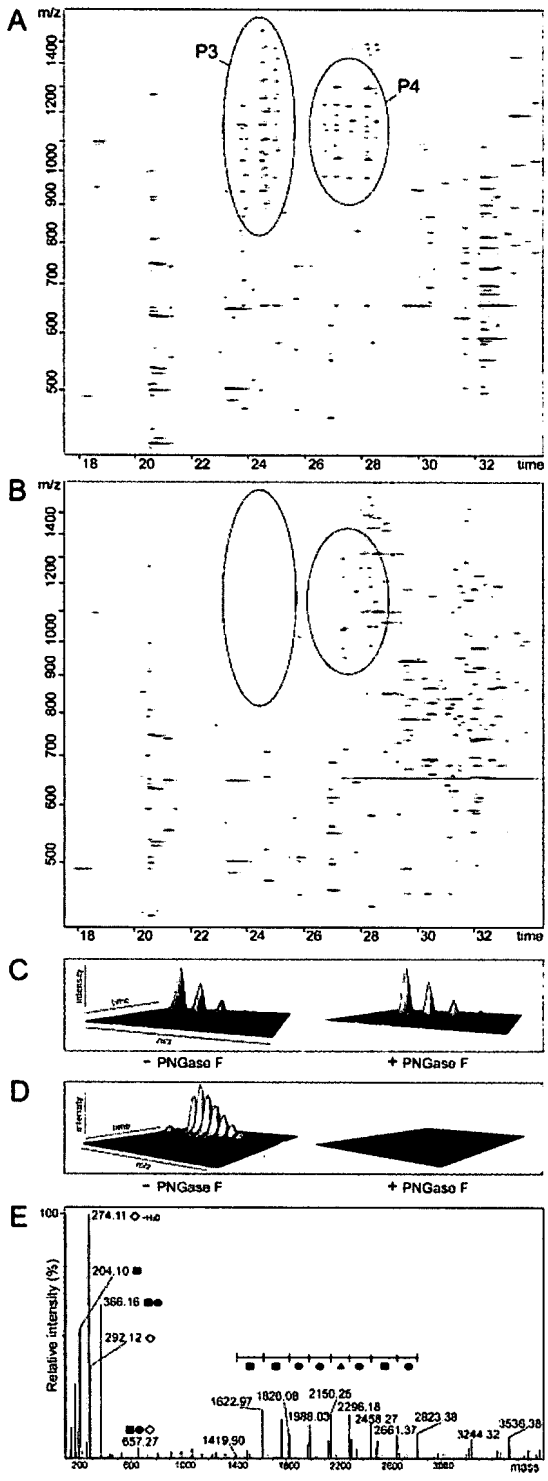


Fig. 1. Visualization of tryptic peptides of hCGβ by LC–MS. Panel **A** shows LC–MS separation of tryptic peptides of hCGβ purified from the urine of a testicular cancer patient before and after the PNGase F treatment (panel **B**). Panel **C** visualizes in a three-dimensional mode the tryptic peptide P12 (*m/z* 637.3, $[M + 2H]^{2+}$, MW 1272.5) representing peptides existing in equal amounts in the two digests. A putatively N-glycosylated peptide (*m/z* 943.4, $[M + 4H]^{4+}$, MW 3769.6) absent from the PNGase F-treated digest is visualized in panel **D**. Panel **E** shows the MS/MS fragmentation spectra of $[M + 3H]^{3+}$ precursor ion *m/z* 1257.5 (MW 3769.6) and the sugar-specific oxonium ions as well as oligosaccharide fragmentation patterns. Symbols: ■, HexNAc; ●, Hex; ▲, Fuc; ◇, NeuAc.

tetra-antennary glycans and some Asn-30-linked oligosaccharides lacking fucose were observed.

Site-specific O-glycan analysis of hCGβ

The O-glycosylated tryptic peptides were difficult to detect by LC–MS analysis of the crude tryptic digest probably because of suppression by the enormous heterogeneity of *N*-glycan containing peptides. The C-terminal portion of hCGβ was therefore isolated by RP-high performance liquid chromatography (HPLC) after cleavage with GluC protease. The N-terminus of the fragment was confirmed to be the expected Ser-66 by LC–MSMS analysis of the fragment tryptic digest (data not shown).

Direct ESI–MS analysis of the purified C-terminal fragment showed a wide variety of heterogeneous masses. The deconvulated masses of the C-terminal hCGβ fragment from normal late pregnancy, invasive mole, and testicular cancer are shown in Figure 4. On the basis of the masses observed, it was evident that sialylated core-2 structures were present at all four O-glycosylation sites in hCGβ purified from a testicular cancer patient (Figure 4D), whereas both core-1- and core-2-containing structures were observed in molar disease and pregnancy. The small masses of the C-terminal peptide in pregnancy hCG (Figure 4A) showed that variants with some unoccupied O-glycosylation sites also existed.

The site-specific *O*-glycan structures were studied in glycopeptides produced by tryptic digestion of the C-terminal fragment. Peptides P13 and P15, which contained Ser-121- and Ser-138-linked oligosaccharides, respectively, were almost exclusively present as $[M + 2H]^{2+}$. The major oligosaccharide structures assigned by the mass-matching approach are shown in Figure 5. Ser-121 was constantly occupied by a core-2 glycan, whereas the core-1 structure always dominated at Ser-138, with some core-2 structure observed in malignancy. Both sites were also observed unoccupied by any glycan. The possibility that the exposure of small Tn and T antigens within these sites could be due to the desialylation in the mass spectrometric analysis cannot be ruled out.

Analysis of P14 was complicated because of the presence of glycans on both Ser-127 and Ser-132 within the peptide. Figure 5C shows the observed masses, the major possible glycans, and combinations of these in four groups: (1) one core-1, (2) two core-1 or one core-2, (3) one core-2 and one core-1, or (4) two core-2 *O*-glycans. A single oligosaccharide was detected in most of the P14 from pregnancy and invasive mole; thus, either Ser-127 or Ser-132 was not glycosylated in almost half of the hCGβ molecules. Either two core-1 glycans or one core-2 glycan made up most of the rest. These two had the same mass and could not be differentiated by the mass-matching approach. The largest mass of this group (2417.0) consists of either two intact core-1 structures with one sialic acid or one sialylated core-2 structure. The intense masses of 2126.0 and 1963.9 lacked either one sialic acid or, in addition, one hexose, respectively. In larger glycopeptides, visible mainly in P14 from testicular cancer, both glycosylation sites were occupied, but because different core-1 and core-2 structures could result in the same glycopeptide mass, the interpretation was difficult. However, the major structure (3073.3) contained a sialylated core-2

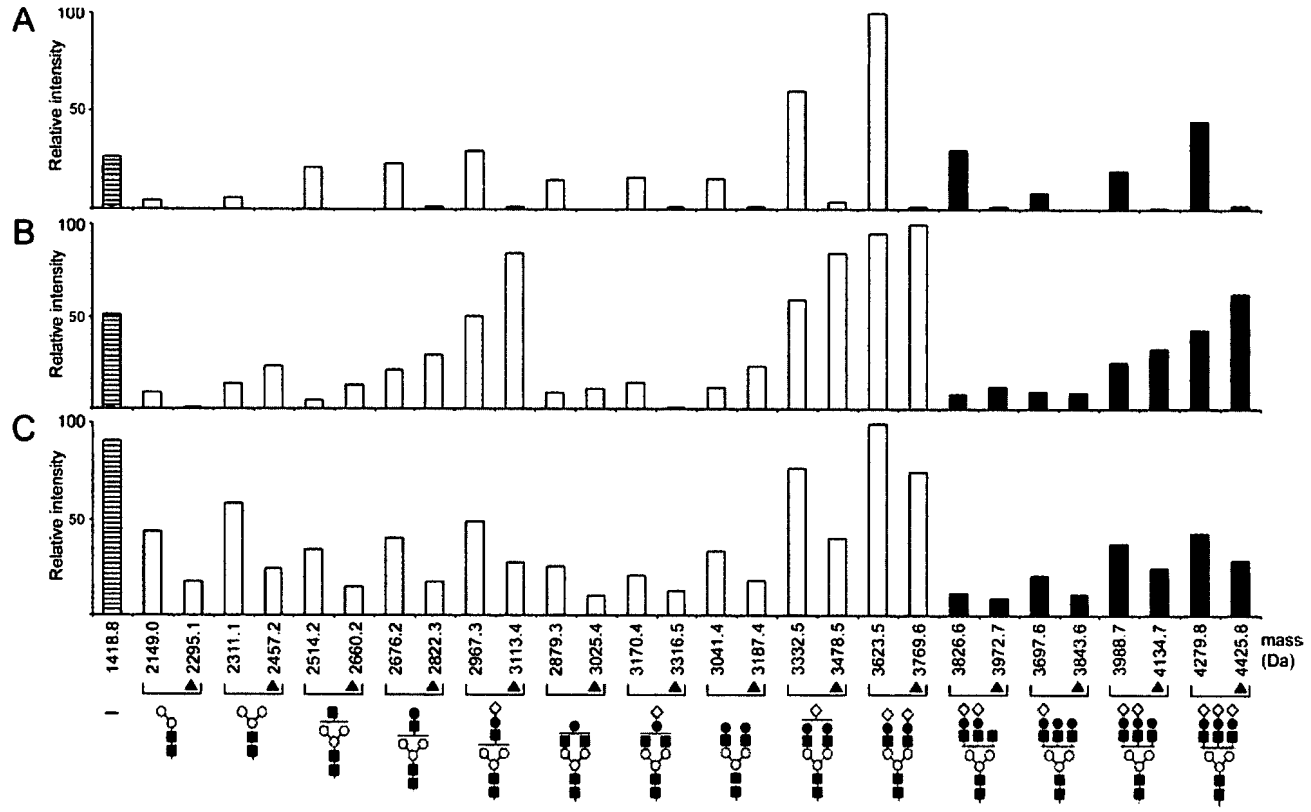


Fig. 2. Structures and relative intensities of the glycans attached to Asn-13 of hCGβ. Panel A shows P3 containing various oligosaccharides from the urine of a normally pregnant woman at 35 weeks of pregnancy, panel **B** from the urine of a patient suffering from invasive mole, and panel **C** from the urine of a patient with testicular cancer. The proposed sugar structures have been calculated by the mass-matching approach, where the theoretical polypeptide mass of P3 is subtracted from the observed masses. Triantennary glycan structures are shown in black, biantennary in gray, monoantennary in white, and the peptide without glycan is shown with lines. Symbols: ■, GlcNAc; ○, Man; ●, Gal; ▲, Fuc; ◇, NeuAc. Fuc is attached to the first GlcNAc within the structures when present.

structure in one and an intact core-1 structure with one sialic acid in the other site. The glycopeptide with a mass of 3729.5 contained two sialylated core-2 oligosaccharides.

Differential glycan analysis of hCGβ in malignancy and pregnancy

Characteristic site-specific differences and similarities in the glycan structures were observed between hCGβ from patients with testicular cancer, choriocarcinoma, invasive mole, pregnant women of different gestational age, and a pharmaceutical hCG preparation and hCG from JEG-3 cells (Table III). The oligosaccharide structures linked to Asn-13 showed no significant increase of triantennary structures in malignancy, whereas both triantennary and monoantennary oligosaccharides attached to Asn-30 were enriched in hCG from cancer patients. The degree of fucosylation in the two N-glycosylation sites varied. The Asn-30-linked oligosaccharides were mainly fucosylated, whereas fucosylation of Asn-13-linked glycans varied (Table III). Less than 25% of the oligosaccharides were fucosylated in hCGβ from late pregnancy and JEG cells, whereas the fucosylation degree exceeded 36% in hCGβ from malignancies.

Ser-121 in P13 contained a core-2 *O*-glycan structure irrespective of the source of hCG. In contrast, core-1 oligosaccharide structures containing either one or two sialic acids dominated at Ser-138. Especially in hCGβ from testicular cancer, but also to some extent in early pregnancy and JEG-3 cells, the core-2 structure was observed (Figure 5, Table III).

The two O-glycosylation sites of P14 displayed the largest variation between different hCGβ preparations. In pregnancy and molar disease, a single core-1 structure was most common, with either one core-2 structure or two core-1 structures as the second most common form. In cancer patients and early pregnancy, core-2 oligosaccharide structures were most often present in either one or both sites. The glycan composition of the commercial hCG preparation resembled that in early pregnancy urine.

Fairly large individual differences were observed between the two samples from late pregnancy and the two testicular cancer patients. Thus, the patterns observed in this study are likely to represent only part of the true diversity.

Glycan analysis of hCGβ variants isolated from a single patient

Various forms of hCG were isolated from the patient with an invasive mole for site-specific glycan analysis. hCG was purified both from urine and from serum, and the hCGβ subunit was dissociated from the heterodimer. Very similar oligosaccharide structures and the same polypeptide chain cleavages in P5 and P9 occurred in hCG from serum and urine. In urinary hCGh isolated with monoclonal antibody (MAb) B152, core-2 *O*-glycans dominated in P14, and only 1% contained a single core-1 glycan. Interestingly, no *N*-glycan structure was

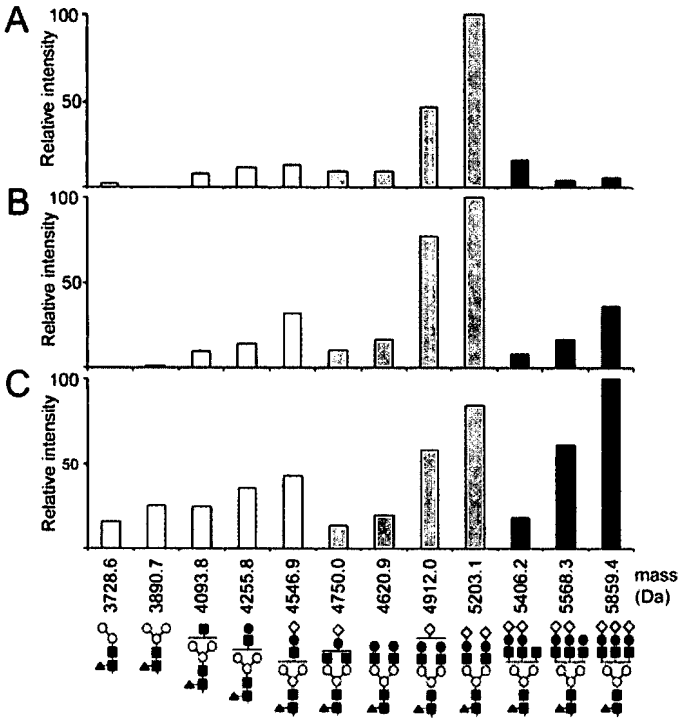


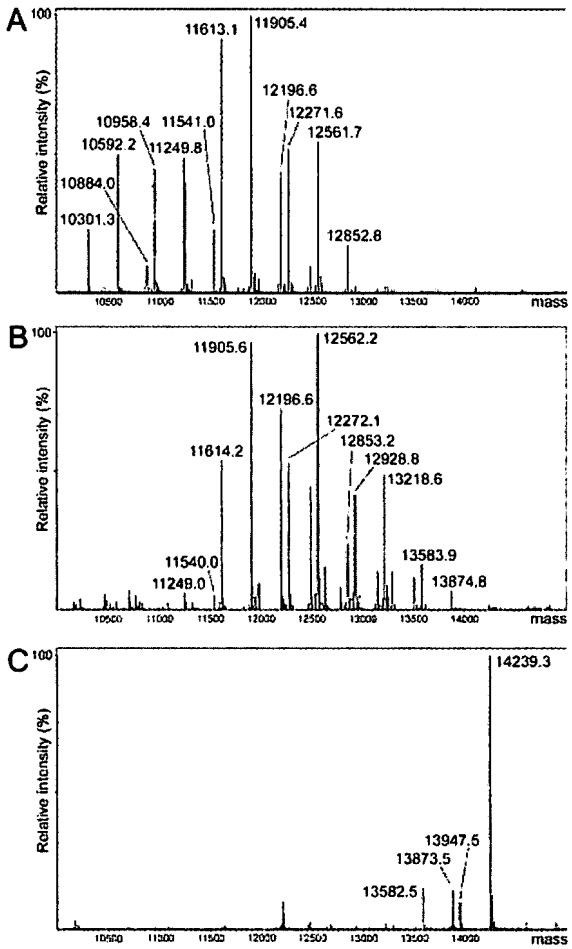
Fig. 3. Structures and relative intensities of the glycans attached to Asn-30 of hCGβ. P4 containing oligosaccharides purified from the urine of a pregnant woman is shown in panel **A**, from the urine of an invasive mole patient in panel **B**, and from the urine of a testicular cancer patient in panel **C**. The proposed sugar structures are calculated by the mass-matching approach. Triantennary glycan structures are shown in black, biantennary in gray, and monoantennary in white. Symbols are as in Figure 2.

enriched. Free hCGβ isolated from urine of this patient contained more triantennary *N*-glycans in P3 and P4 and more core-2 *O*-glycans in P14 than that in hCG, and a higher proportion of the Asn-13 glycan was fucosylated (Table IV).

Discussion

This study confirms the results of earlier ones (Elliott *et al.*, 1997; Kobata and Takeuchi, 1999), showing differences in the carbohydrate structure of hCGβ derived from pregnancy and malignant tumors, respectively. Furthermore, we demonstrate large differences in glycan structures between the different glycosylation sites and in hCG from various sources, which have not been characterized before.

We used an ESI–MS connected to an LC to analyze site-specific glycan structures in tryptic peptides of hCGβ. Furthermore, we used novel differential expression analysis software, designed for proteomics (Skold *et al.*, 2002), to study differential abundance of oligosaccharide structures in the protein. Glycopeptides were identified by comparing the tryptic digest of a protein with a digest of the same deglycosylated molecule. Digests of the same protein purified from different sources and different isoforms of the same protein could also be compared. The combination of LC–MS analysis with differential expression analysis is especially useful for glycoproteins that, such as hCG, are relatively easily digestible with proteases despite the oligosaccharides



D

	Theoretical	Observed		
	m (Da)	Preg 35w	Mole	TestisCA
hCGβ C-terminus (aa 66-145)[a]	6888.3			
+ $HexNAc_2Hex_2NeuAc_2$	10300.8	x		
+ $HexNAc_2Hex_2NeuAc_3$	10591.9	x		
+ $HexNAc_2Hex_2NeuAc_4$	10883.0	x		
+ $HexNAc_3Hex_3NeuAc_3$	10957.0	x		
+ $HexNAc_3Hex_3NeuAc_4$	11248.1	x	x	
+ $HexNAc_3Hex_3NeuAc_5$	11539.2	x	x	
+ $HexNAc_4Hex_4NeuAc_4$	11612.5	x	x	
+ $HexNAc_4Hex_4NeuAc_5$	11803.8	**x**	x	
+ $HexNAc_4Hex_4NeuAc_6$	12195.1	x	x	
+ $HexNAc_5Hex_6NeuAc_5$	12271.3	x	x	
+ $HexNAc_5Hex_5NeuAc_6$	12560.4	x	**x**	
+ $HexNAc_5Hex_6NeuAc_7$	12851.7	x	x	
+ $HexNAc_6Hex_6NeuAc_6$	12927.9		x	
+ $HexNAc_6Hex_6NeuAc_7$	13218.8		x	
+ $HexNAc_7Hex_7NeuAc_7$	13581.9		x	x
+ $HexNAc_7Hex_7NeuAc_8$	13873.0		x	x
+ $HexNAc_8Hex_8NeuAc_7$	13946.9			x
+ $HexNAc_8Hex_8NeuAc_8$	14238.2			**x**

[a] ethylpyridyl-modified cysteines

Fig. 4. ESI–MS analysis of the C-terminal part of hCGβ. The deconvoluted spectra of GluC-cleaved hCGβ C-terminal fragment from the urine of a pregnant woman (**A**), an invasive mole patient (**B**), and a testicular cancer patient (**C**) are shown. Panel **D** indicates the theoretical masses of different monosaccharide compositions when attached to the C-terminal polypeptide and visualizes their matches to the observed masses. The most intense mass observed in different samples is shown in boldface type.

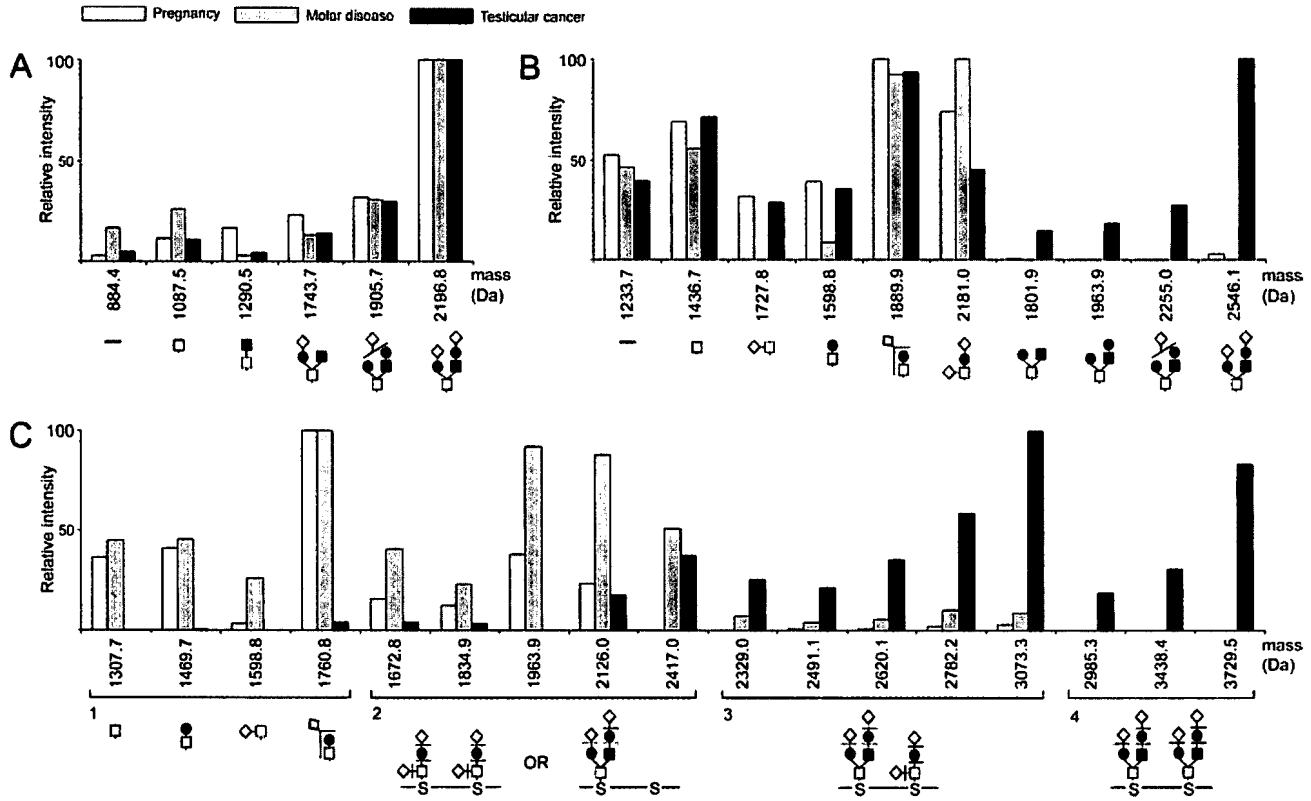


Fig. 5. Structures and relative intensities of the glycans attached to Ser-121, Ser-127, Ser-132, and Ser-138 of the hCGβ. P13 containing oligosaccharides is shown in **A**, P15 containing oligosaccharides in **B**, and P14 containing structures in **C**. The glycans of hCGβ purified from the urine of a pregnant woman are visualized in white, from the urine of an invasive mole patient in gray, and from the urine of a testicular cancer patient in black. The proposed sugar structures attached to Ser-121 (**A**) and Ser-138 (**B**) are calculated by the mass-matching approach. In panel **C**, putative structures attached to P14 are clustered into four categories to represent different sugar structures capable of occurring in Ser-127 and Ser-132: (1) one core-1, (2) two core-1 or one core-2, (3) one core-2 and one core-1, or (4) two core-2 glycans. Proposed structures are gathered under the vertical lines pointing to the masses capable of representing the structures. Symbols: □, GalNAc; ■, GlcNAc; ●, Gal; ◇, NeuAc.

attached. This approach relies entirely on mass matching, and it does not provide exact linkage information about the glycans. However, the glycan structures of hCG have been fairly extensively studied by enzymatic deglycosylation combined with mass spectrometric analysis, and on the basis of this information, it was possible to deduce structures solely by mass matching.

The N-glycosylation of hCG has been reported to be different in choriocarcinoma than in pregnancy (Mizuochi *et al.*, 1983; Elliott *et al.*, 1997), but site-specific glycan differences of hCG have not been studied. In this study, the earlier observed increase in the content of triantennary complex-type *N*-glycans in choriocarcinoma hCG (Elliott *et al.*, 1997) was seen only in the oligosaccharides attached to Asn-30 but not in that attached to Asn-13. The earlier described increase in monoantennary *N*-glycans (Elliott *et al.*, 1997) was observed in sugar structures attached to both Asn-13 and Asn-30. The abnormal biantennary *N*-glycan structures bearing both antennae within the same mannose (Man) residue detected in choriocarcinoma by Kobata *et al.* (Mizuochi *et al.*, 1983; Kobata and Takeuchi, 1999) could not be detected by the mass-matching approach because these structures carry the same mass as ordinary biantennary *N*-glycans. Another site-specific difference between the two *N*-glycans was the degree of fucosylation. Asn-30-linked glycans were nearly totally fucosylated, as earlier reported for pregnancy hCG (Kessler, Reddy, *et al.*, 1979; Weisshaar *et al.*, 1991), whereas the fucosylation degree of Asn-13-linked oligosaccharides varied between 2 and 56%. Furthermore, fucosylation on Asn-13-attached glycans was enriched in malignancies, and it was also higher in early than in late pregnancy. An increased fucose content of *N*-glycans in choriocarcinoma hCG with no site specificity has earlier been reported in one study (Mizuochi *et al.*, 1983) but not in another one (Elliott *et al.*, 1997).

Increased *N*-glycan fucosylation has previously been reported to occur in α-fetoprotein produced by hepatocellular carcinoma, and this is of diagnostic value (Aoyagi *et al.*, 1985). Fucose on the first GlcNAc residue of complex-type *N*-glycans can be specifically recognized by *Lens culinaris* agglutinin (LCA) lectin. Lectins have also been used to distinguish glycosylation differences in hCG from pregnancy and choriocarcinoma cell lines, but LCA was not among the lectins studied (Kelly *et al.*, 2005).

Reports on the O-glycosylation of hCGβ are controversial (Kessler, Mise, *et al.*, 1979; Liu and Bowers, 1997; Birken *et al.*, 2003; Gervais *et al.*, 2003) and may be explained by the site-specific *O*-glycan difference observed in the present study. The core-1 structure has been found to dominate in pregnancy hCG, but we found that Ser-121 always contained a

Table III. Glycan composition of hCG isolated from urine of two testicular cancer patients with stage 1 (TCa1) and stage 3 disease (TCa3,), one choriocarcinoma patient (ChCa), one patient with an invasive mole (Mole), and from pregnant (P) women at different times of gestation. A commercially available preparation of urinary hCG (Pregn) and hCG isolated from the spent medium from a choriocarcinoma cell line (JEG) are shown

	TCa1	TCa3	ChCa	JEG	Mole	P 5 weeks	P 7 weeks	P 35 weeks	P 35 weeks	Pregn
P3 (Asn-13) without glycan	7.7	2.5	1.4	3.9	5.1	1.9	4.1	5.5	4.3	2.7
+ Monoantennary	32.5	32.6	20.0	33.3	26.9	30.1	21.7	22.6	11.9	18.3
+ Biantennary	36.1	44.2	61.5	52.1	40.7	47.2	59.9	44.6	57.2	65.7
+ Triantennary	20.0	17.5	15.9	10.7	24.0	18.3	14.0	26.4	25.6	12.7
+ Tetra-antennary	3.6	3.1	1.2	0	3.4	2.5	0.2	1.0	0.9	0.6
P4 (Asn-30) without glycan	1.0	0.4	0	0	1.1	0.4	0.2	0.6	0.5	0.8
+ Monoantennary	33.5	23.9	4.4	4.1	18.9	15.3	15.9	18.2	11.8	12.0
+ Biantennary	33.7	36.9	81.8	90.7	60.6	67.0	78.4	72.2	76.5	83.9
+ Triantennary	29.7	38.1	13.2	5.2	19.4	17.3	5.5	9.0	11.1	3.2
+ Tetra-antennary	2.0	0.7	0.6	0	0	0	0	0	0.1	0
P13 (Ser-121) without glycan	2.7	2.9	2.1	0.8	7.2	1.7	2.4	8.0	1.3	3.1
+ Core-1	12.9	13.2	11.5	3.6	24.1	9.1	12.7	23.7	14.2	29.3
+ Core-2	84.4	83.9	86.4	95.6	68.7	89.2	84.9	68.3	84.5	67.7
P14 (Ser-127 and Ser-132) without glycan	0	0	0	0	0	1.0	2.5	6.4	2.5	0.7
+ 1 core-1	0.8	1.9	2.5	1.9	40.0	36.0	68.1	91.6	62.1	24.5
+ 2 core-1 or 1 core-2	13.2	29.7	66.5	40.4	53.5	26.2	18.5	2.0	32.5	52.4
+ 1 core-2 and 1 core-1	49.8	55.9	29.3	48.9	6.5	30.2	10.8	0	2.9	20.7
+ 2 core-2	36.1	12.5	1.7	8.8	0	6.6	0	0	0	1.7
P15 (Ser-138) without glycan	8.0	21.1	30.9	10.4	15.4	13.3	14.2	25.0	14.2	9.1
+ Core-1	55.1	67.2	64.8	69.0	84.6	70.7	81.6	69.6	84.9	82.0
+ Core-2	36.8	11.7	4.3	20.7	0	16.0	4.1	5.4	0.9	8.9
P3 glycans										
– Fucose	63.6	53.2	58.2	86.8	43.7	63.6	76.5	96.4	76.5	73.9
+ Fucose	36.4	46.8	41.8	13.2	56.3	36.4	23.5	3.6	23.5	26.1
P4 glycans										
– Fucose	15.2	4.3	1.5	18.3	0	4.1	12.0	21.4	5.8	13.2
+ Fucose	84.8	95.7	98.5	81.7	100.0	95.9	88.0	78.6	94.2	86.8

The proportions of different oligosaccharide structures present on P3, P4, P13, P14, and P15 were calculated on the basis of the relative intensities of glycopeptides detected by LC–MS. The degree of fucosylation of the *N*-glycans in P3 and P4 is shown. P3 and P4 contain one *N*-glycan each, P13 and P15 one *O*-glycan each, and P14 two *O*-glycans.

biantennary core-2 structure. This was previously suggested (Liu and Bowers, 1997) but was not found in the original reports (Kessler, Mise, *et al.*, 1979). The structure of this glycan was virtually identical in all the conditions studied. The oligosaccharide attached to Ser-138 was, on the contrary, in all samples studied mainly of core-1 type, with either one or two sialic acids attached. Core-2 structures were observed only in hCGβ purified from one testicular cancer patient, the choriocarcinoma cell line, and very early pregnancy.

The occurrence of two O-glycosylation sites in P14, which is not specifically cleavable by known proteases, complicated the analysis of the oligosaccharide structures in Ser-127 and Ser-132. Despite this, it was evident that in pregnancy, only a single glycosylation site was occupied in a significant proportion of hCG, as suggested earlier (Liu and Bowers, 1997). In most malignancy-derived hCG, two glycosylation sites were occupied, and two core-2 oligosaccharides were often detected. This is in agreement with the observation that MAb B152 recognizes a Ser-132-linked core-2 structure (Birken *et al.*, 2003). The O-glycosylation of hCG purified from urine of a patient with an invasive mole resembled pregnancy-derived hCG, whereas the N-glycosylation pattern was more similar to that in cancer-derived hCG. In very early pregnancy, the glycosylation of P14 resembled that in cancer in that both sites were occupied. Some unique *O*-glycan structures have been observed in recombinant hCG (Gervais *et al.*, 2003), including *O*-glycan fucosylation and increased content of *N*-acetylhexosamine. These structures were not observed in the human hCG preparations analyzed in this study. Thus, they appear to represent oligosaccharides specific to the Chinese hamster ovary cell line used to express recombinant hCG.

In addition to glycans observed in the study, it is interesting to note that some glycosylation sites, notably Ser-138, Ser-121, and Asn-13, are not glycosylated in some of the hCG variants studied.

Table IV. Glycan composition of various forms of hCGβ isolated from urine of a patient with hydatidiform mole

	U-hCG	U-hCGβ	U-hCGh	S-hCG
P3 (Asn-13) without glycan	5.1	0.5	3.4	2.2
+ Monoantennary	26.9	7.9	26.1	20.8
+ Biantennary	40.7	45.4	46.3	53.9
+ Triantennary	24.0	37.2	21.8	19.8
+ Tetra-antennary	3.4	8.9	2.3	3.3
P4 (Asn-30) without glycan	1.1	0	0.7	0.3
+ Monoantennary	18.9	4.2	15.4	13.6
+ Biantennary	60.6	61.4	55.6	65.3
+ Triantennary	19.4	30.0	28.3	19.8
+ Tetra-antennary	0.0	4.4	0	1.0
P13 (Ser-121) without glycan	7.2	2.2	1.0	2.1
+ Core-1	24.1	12.9	3.4	16.5
+ Core-2	68.7	85.0	95.6	81.4
P14 (Ser-127 and Ser-132) without glycan	0	0	0	3.5
+ 1 core-1	40.0	13.8	1.0	63.7
+ 2 core-1 or 1 core-2	53.5	36.5	20.3	20.3
+ 1 core-2 and 1 core-1	6.5	49.7	73.9	11.1
+ 2 core-2	0	0	4.8	1.4
P15 (Ser-138) without glycan	15.4	15.4	26.9	21.8
+ Core-1	84.6	78.7	73.1	74.9
+ Core-2	0	5.9	0	3.3
P3 glycans				
– Fucose	43.7	16.4	41.0	50.0
+ Fucose	56.3	83.6	59.0	50.0
P4 glycans				
– Fucose	0	0	2.5	3.9
+ Fucose	100.0	100.0	97.5	96.1

The percentages of the main structures present and the degree of fucosylation on the *N*-glycans are shown. Intact heterodimeric hCG (U-hCG), free β-subunit (U-hCGβ), and "hyperglycosylated" hCG (U-hCGh) were purified from urine and heterodimeric hCG also from serum (S-hCG). P3 and P4 contain one *N*-glycan each, P13 and P15 one *O*-glycan each, and P14 two *O*-glycans.

Most studies on hCG glycosylation have been performed on hCG prepared from pooled pregnancy urine (Kessler, Mise, *et al.*, 1979; Kessler, Reddy, *et al.*, 1979; Weisshaar *et al.*, 1991; Laidler *et al.*, 1995; Liu and Bowers, 1997), and thus, the glycan structures observed can be expected to represent the average oligosaccharide composition of pregnancy hCG. In the commercial hCG preparation used in the present study, a fairly high proportion of core-2-associated P14 was observed, indicating that the preparation was mainly derived from first-trimester urine. The choriocarcinoma cell line JEG-3 is a potential source of an hCGh standard for assays based on MAb B152. The *N*-glycan structures of JEG-3-derived hCG resembled that from early pregnancy, including the degree of fucosylation and the content of triantennary glycan structures. The proportion of core-2 structures in P14 and P15 was as high as in hCG from cancer patients. This explains the strong reactivity of malignancy-related hCG with MAb B152.

Various isoforms of hCG, including free hCGβ and hCGh, have been shown to be associated with malignancies (Alfthan, Haglund, Dabek, *et al.*, 1992; Alfthan, Haglund, Roberts, *et al.*, 1992; Elliott *et al.*, 1997). We, therefore, isolated free hCGβ and hCGh from urine and purified hCG from serum and urine of a patient with an invasive mole. Interestingly, the oligosaccharides of hCG isolated from serum displayed the same glycan pattern as hCG purified from urine. Therefore, the heterogeneous oligosaccharide structures of urinary hCG are not modified by additional degradation in urine. In hCGh isolated with MAb B152, an increase in core-2-containing P14 was seen, but no other difference in glycan composition, except a small increase in triantennary Asn-30-associated glycan, was observed. The degree of fucosylation in the Asn-13-linked glycan was not changed. Therefore, the hCGh detected by MAb B152 differs markedly from "normal" hCG only with respect to the Ser-132-associated glycan. This confirms the finding that B152 recognizes this core-2 *O*-glycan and surrounding polypeptide structures (Birken *et al.*, 2003).

Interestingly, the oligosaccharide structures of free hCGβ resembled that of hCGh with a high proportion of

triantennary N-linked glycans and core-2 type *O*-glycans. In addition, the fucosylation of the Asn-13-linked glycan was markedly elevated. This observation could indicate that the large glycans might inhibit the association of hCGβ with the α-subunit. The observation of malignancy-related "hyperglycosylation" of free hCGβ is in agreement with the fact that the proportion of hCGβ is increased in patients with trophoblastic cancer (Alfthan, Haglund, Dabek, *et al.*, 1992; Alfthan, Haglund, Roberts, *et al.*, 1992). Because fucosylated *N*-glycans react with LCA lectin, the malignancy-associated hCGβ could possibly be detected with a lectin assay.

In conclusion, we characterized six site-specific glycan structures of hCG by differential expression analysis of LC–MS data on hCG isolated from pregnant women and patients with malignant diseases. Major differences were observed in the fucosylation degree of Asn-13-linked glycans as well as in the occurrence of core-2 *O*-glycans in Ser-127, Ser-132, and Ser-138. Furthermore, larger glycan structures and a higher degree of fucosylation of Asn-13-linked glycans were observed in free hCGβ than in heterodimeric hCG from the same patient. Analysis of hCGh isolated by affinity chromatography with MAb B152 confirmed that this antibody recognizes a core-2 glycan on Ser-132 and on the surrounding peptide structures.

Materials and methods

Patients and samples

hCG was isolated from the urine of two patients with non-seminomatous testicular cancer (stages 1 and 3, respectively) (Sobin and Wittekind, 2002), one with choriocarcinoma (stage IIIA) and one with an invasive mole (stage IB) (Benedet and Pecorell, 2000). Urine from one pregnant woman was collected at 5 and 7 weeks and from two women at 35 weeks after fertilization. Informed consent was obtained from all patients, and the study was approved by the institutional ethics committee.

NaN_3 was added to a concentration of 0.5 g/L into the urine samples, which were stored at 4°C. hCG in the samples was quantitated by a time-resolved immunofluorometric assay performed as described (Pettersson *et al.*, 1983; Alfthan, Haglund, Dabek, *et al.*, 1992; Alfthan, Haglund, Roberts, *et al.*, 1992).

The choriocarcinoma cell line, JEG-3 [American Type Culture Collections (ATCC)], was cultured according to the guidelines provided. The hCG-containing medium was centrifuged to remove cells and stored at −20°C. Pregnyl was purchased from Organon Technica, and lot 167823 was used for analysis.

Purification of hCG

hCG was purified from 100 to 1000 mL of urine, containing 3–250 nmol/L of hCG. Urine was clarified by filtering through Nalgene disposable filter unit with 0.45-μm pore size, and the filtrate was applied to an anti-hCG affinity column (1 mL) with a flow rate of 200 μL/min. MAb 6G5, raised in the laboratory as described earlier (Alfthan, Haglund, Dabek, *et al.*, 1992; Alfthan, Haglund, Roberts, *et al.*, 1992), was coupled with CNBr-activated Sepharose 4B (Pharmacia) according to the instructions of the manufacturer. Before immunoaffinity chromatography, the samples were passed through a precolumn of equal size with an unrelated MAb. The columns were equilibrated with 50 mmol/L sodium phosphate (pH 7.4). After sample application, the column was washed with 40 mL of 10 mmol/L ammonium acetate (pH 4.5) and the bound protein eluted with 15 mL of 3 mol/L acetic acid. Fractions of 1 mL were collected and immediately neutralized with NH_4OH. Fractions containing hCG were pooled, concentrated using a Centricon centrifugal device with a cutoff of 10 kDa (Millipore), and fractionated by gel filtration on a Sephacryl S-100 HR (Amersham Biosciences) 16 × 700 mm column in 0.1 mol/L ammonium bicarbonate (pH 8) with a flow rate of 200 μL/min. The content of intact hCG dimer and hCGβ in 800-μL fractions was determined by time-resolved immunofluorometric assays, and the fractions containing hCG were freeze-dried and stored at −20°C.

To separate free hCGβ from hCG, the urine was first applied to an affinity column containing MAb 9C11, raised in the laboratory, and shown to be specific to free hCGβ (Alfthan, Haglund, Dabek, *et al.*, 1992; Alfthan, Haglund, Roberts, *et al.*, 1992). Bound hCGβ was eluted and further treated as described above. The flowthrough fraction was further applied to the anti-hCG affinity column containing MAb 6G5. hCGh was purified from urine by immunoaffinity chromatography with MAb B152 (Birken *et al.*, 1999) column.

hCG was purified from a 5-mL serum sample from a patient with invasive mole containing 1800 nmol/L of hCG. The sample was first purified by gel filtration on a 16 × 700 mm column packed with Sephacryl S-200 HR (Amersham Biosciences) in 0.1 mol/L ammonium bicarbonate (pH 8) at flow rate of 250 μL/min. Fractions containing proteins <70 kDa were collected and subjected to immunoaffinity purification as described above. hCG was purified from the growth medium of JEG-3 cell line using MAb 6G5.

In-liquid alkylation and digestion of hCGβ

Purified hCG was reduced with dithiotreitol and alkylated with 4-vinyl pyridine (Aldrich) in 6 mol/L guanidine hydrochloride, 2 mmol/L EDTA, 0.5 M Tris (pH 7.5). The alkylated hCG with dissociated α- and β-subunits was desalted by RP-HPLC on a C4 column (symmetry C4, 3.9 × 20 mm, 300 Å, 5 μm, Waters) and eluted with a linear gradient of acetonitrile (0–80% in 30 min) in 0.1% trifluoroacetic acid (TFA). hCGβ-containing fractions were pooled, dried, and subjected to trypsin digestion using 10% w/w sequencing grade trypsin (Promega) in 10 mmol/L ammonium bicarbonate at 37°C for 16 h.

For digestion with Glu-C (V8, Roche), 5% w/w protease was added in 50 mmol/L ammonium acetate (pH 4), and digestion was carried out for 4 h at room temperature. After each incubation hour, 2% w/w V8 was added. The C-terminal portion of hCG was separated by RP chromatography on a C4 column (symmetry C4, 2 × 50 mm, 300 Å, 3.5 μm, Waters). Proteolytic peptides were eluted with a linear gradient of acetonitrile (0–80% in 30 min) in 0.1% TFA with a flow rate of 0.2 mL/min. Fractions containing the C-terminal portion of hCGβ were collected.

For the removal of *N*-glycans, 0.5 U recombinant PNGase F (Roche) was added to 10 μg of hCG in 0.1 M sodium phosphate buffer (pH 7.8). Deglycosylation was carried out for 48 h at 37°C. Another 0.5 U of PNGase F was added after 24-h incubation. The removal of *N*-glycans was confirmed by SDS–PAGE in 12% w/v gel (Laemmli, 1970).

MS

For LC–MS analysis, the peptides were separated by RP-HPLC on a CapLC instrument (Waters) with a 0.075 × 150-mm C18 column (Atlantis dC18, 100 Å, 3 μm, Waters), which was eluted with a linear gradient of acetonitrile (5–50% in 30 min) in 0.1% formic acid. The flow rate was 0.3 μL/min, and the eluent was directly injected into a quadrupole/time-of-flight hybrid mass spectrometer (Q-TOF Micro, Waters) equipped with an ESI source. The mass spectrometer was calibrated using 2 pmol/μL of glufibrinogenic peptide B fragments as a standard.

MSMS fragmentation spectra of the peptides were acquired by colliding the doubly or triply charged precursor ions with argon collision gas at accelerating voltages of 30–45 V.

For ESI–MS analysis of the chromatographically purified C-terminal fragment of hCGβ, the polypeptide was injected into the mass spectrometer (Q-TOF Micro, Waters) via a nanoflow interface with a Hamilton-syringe pump with a flow rate of 0.3 μL/min. The mass spectrometer was calibrated using 400 fmol/μL of myoglobin (Sigma) as a standard.

Differential data analysis

Mass spectra collected during the LC–MS separation of hCGβ peptides were exported into ASCII text files using the DataBridge of the MassLynx software (Waters). The text files were imported into DeCyder MS software (GE Healthcare), where different elution profiles were visualized as 2D maps with *m/z* on the *y*-axis and retention time on the *x*-axis as well as three-dimensional (3D) graphs with intensity based on ion count as *z*-axis. Peptide eluting at specific time point and represented by many different charge states was detected automatically, its charge states were assigned, MS signal ion counts of different charge states were integrated, and the actual mass of the peptide was deconvoluted. The ion counts of all different charge states of the same peptide were taken into account to calculate the total intensity of deconvoluted mass in the range of 500–6000 Da. PepMatch module of DeCyder MS software was used to align peptides from different LC–MS runs and for semiquantitative differential analysis of the peptides. Ion counts over the spot areas manually selected were integrated, and comparison of the integrated ion counts between samples was performed.

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Conflict of interest statement

None declared.

Abbreviations

ESI, electrospray ionization; Fuc, fucose; hCG, human chorionic gonadotropin; hCGh, hyperglycosylated hCG; Hex, hexose; HexNAc, *N*-acetylhexosamine; HPLC, high performance liquid chromatography; LC, liquid chromatography; MAb, monoclonal antibody; MS, mass spectrometry; MSMS, tandem mass spectrometry; NeuAc, *N*-acetylneuraminic acid; PNGase F, peptide-*N*-glycosidase F; RP, reverse phase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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